Hepatitis B virus and hepatocellular carcinoma

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Received for publication 24 October 2000
Accepted for publication 13 November 2000

Summary. Chronic hepatitis B virus (HBV) infection is a major global cause of hepatocellular carcinoma (HCC). Individuals who are chronic carriers have a greater than 100-fold increased relative risk of developing the tumour. Several mechanisms of HBV-induced HCC have been proposed. Integration of HBV DNA into the genome of hepatocytes occurs commonly, although integration at cellular sites that are important for regulation of hepatocyte proliferation appears to be a rare event. Functions of the HBx protein are also potentially oncogenic. These include transcriptional activation of cellular growth regulatory genes, modulation of apoptosis and inhibition of nucleotide excision repair of damaged cellular DNA. The effects of HBx are mediated by interaction with cellular proteins and activation of cell signalling pathways. Variations in HBV genome sequences may be important in hepatocarcinogenesis, although their significance has not yet been completely elucidated. Necroinflammatory hepatic disease, which often accompanies chronic HBV infection, may contribute indirectly to hepatocyte transformation in a number of ways, including by facilitating HBV DNA integration, predisposing to the acquisition of cellular mutations and generating mutagenic oxygen reactive species. Although HCC is a malignancy with a poor prognosis, the availability of an effective vaccine against HBV infection, and its inclusion in the Expanded Programme of Immunization of many countries, augurs well for the eventual elimination of HBV-associated HCC.

Keywords: Viral integration, hepatitis Bx protein, mutations, chronic necroinflammatory hepatic disease, immunization

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Introduction

Hepatitis B virus (HBV) was one of the first viruses to be causally linked to a human tumour. Along with tobacco, it is now thought to be the most important environmental carcinogen to which humans are exposed. There are estimated to be 387 million carriers of HBV in the world

today. As many as one-quarter of these will develop hepatocellular carcinoma (HCC). HBV is implicated in the aetiology of as much as 80% of the HCC that occurs with such high frequency in Chinese and black African populations. Furthermore, HBV, hepatitis C virus (HCV) and cirrhosis between them contribute to the genesis of almost all global HCCs. An effective vaccine against HBV has been available for several years and has been included in the Expanded Programme of Immunization in many countries. HBV-induced HCC may thus prove to be the first tumour to be prevented by universal immunization against the responsible virus. Such an achievement would be of momentous public health importance because HCC is one of the major malignant diseases in the world today. HCC ranks fifth in overall frequency, fifth in men and eighth in women, and is the most prevalent or amongst the most prevalent tumours of the populous regions of the world. The annual mortality rate from HCC is virtually the same as its annual incidence, attesting to its rapid course and grave

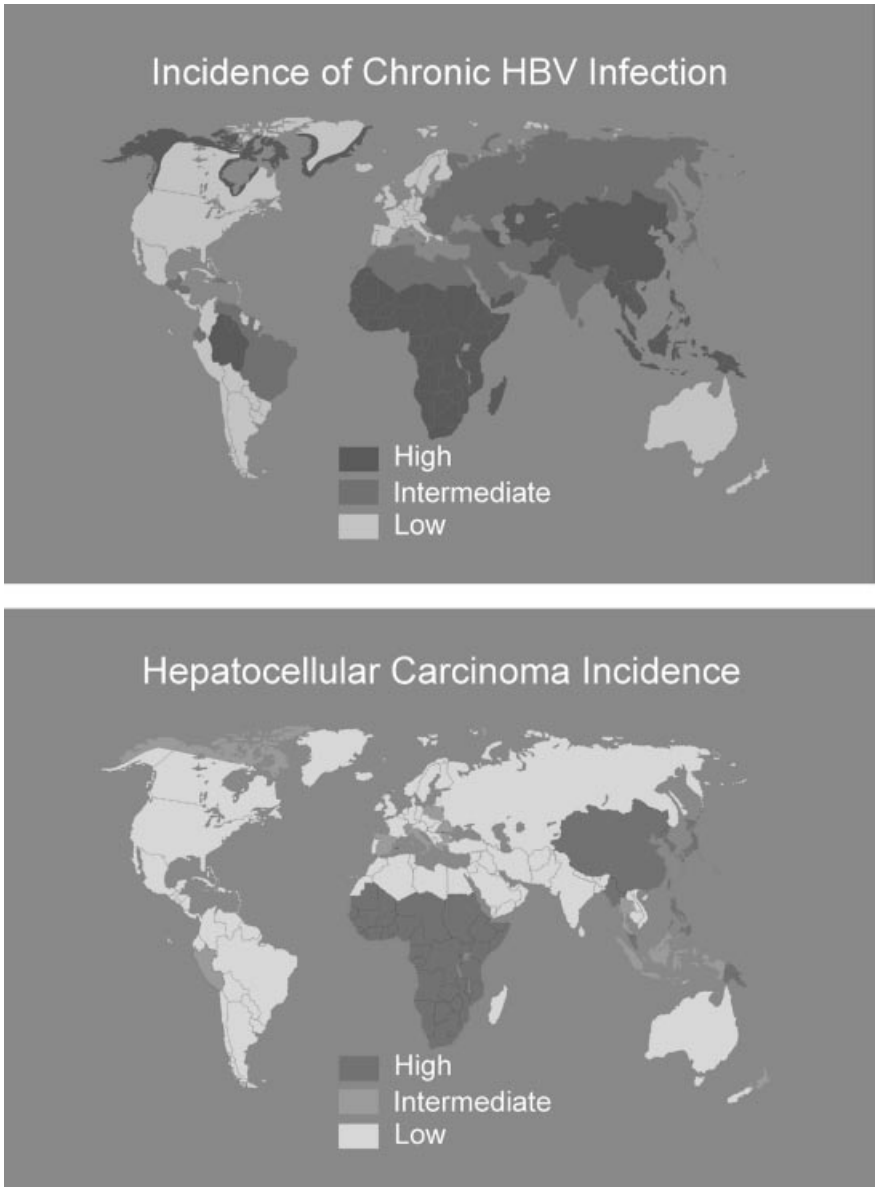

Figure 1. Global maps showing the close similarity between the geographical distributions of chronic hepatitis B virus infection and hepatocellular carcinoma.

prognosis. In populations with a high incidence of the tumour, HCC often occurs in young males and has important economic consequences.

A causal role for HBV in HCC is now beyond doubt, but the exact mechanisms of virus-induced hepatocyte transformation remain uncertain. The focus of this review is an analysis of the epidemiology and molecular biology that provide evidence for the association between chronic HBV infection and HCC.

Evidence for a role of hepatitis B virus in the aetiology of hepatocellular carcinoma

An aetiological association between cirrhosis and HCC was suspected as early as the beginning of the 20th Century (Kew, 2000). By the 1950s it was realized that it was posthepatitic cirrhosis that was most closely linked to tumour formation (Sheldon & James 1948; Walsh & Wolff 1952; Steiner & Davies 1957; Higginson 1963). Following the introduction of serological tests for HBV in the late 1960s it soon became evident that chronic HBV infection was a major risk factor for the development of HCC. Refinements of molecular techniques for identifying viral DNA, availability of HCC cell lines harbouring integrated HBV DNA, and a variety of animal models, either infected with viruses belonging to the same family as HBV (the *hepadnaviridae*) or transgenically propagated with specific HBV genes, has made it possible to investigate in depth the role of chronic HBV infection in the pathogenesis of HCC.

Geographical correlation between the prevalence of hepatitis B virus carriage and hepatocellular carcinoma. With a few exceptions, the global distributions of chronic HBV infection and HCC are closely similar (Figure 1) (Szmuness 1978; Ohta 1976; Kew 1981; IARC. 1994). Countries in eastern and south-eastern Asia, some of the western Pacific islands, and sub-Saharan Africa, with HBV carrier rates of 7–15%, have the highest incidences of HCC (age-adjusted rates as high as 113 per 100 000 of the population per annum) (Prates & Torres 1965; Parkin *et al.* 1993; Bosch 1997; Parkin *et al.* 1997). In industrialized countries, where carrier rates are usually less than 2%, the tumour is uncommon or rare (age-adjusted incidences of less than 5, or even 3, per 100 000 per annum). Countries with intermediate carrier rates (2–7%), such as a number of Far Eastern countries and Greece, have intermediate incidences of the tumour. The incidence of HBV-related HCC may also vary within a given country or area (Harington *et al.* 1975; Trichopoulos *et al.* 1976; Bosch 1997). For example, in The People's Republic of China, HCC occurs most often in the north-eastern province of Jilin and along the south-eastern seaboard (Bosch 1997), and in Mozambique, the tumour is most common in the coastal regions around Inhambane and Morumbene (Harington *et al.* 1975). The geographical correlation between HBV carriage and the occurrence of HCC provided an early intimation that the two may be causally related. Further evidence was provided by familial clustering of patients with HCC who are also chronically infected with HBV (Ohbayashi *et al.* 1972; Tong *et al.* 1979).

For the geographical parallel between HCC and HBV to be absolute would require the virus to be the sole cause of the tumour and that it acts alone. The evidence indicates that HCC is multifactorial in aetiology and pathogenesis. Most exceptions to the close correlations between chronic HBV infection and HCC are explained by the occurrence of HCV- or alcoholic cirrhosis-induced HCC (Tuyns & Obradovic 1975; Kobayashi *et al.* 1994). For example, in Japan, where there is an intermediate incidence of HBV carriage, the high incidence of HCC is almost entirely attributed to persistent HCV infection.

Hepatitis B virus markers in serum and tissues of patients with hepatocellular carcinoma. The first direct evidence of an association between chronic HBV infection and HCC was a report of five patients with HCC whose serum was positive for HBV surface antigen (HBsAg) (Sherlock *et al.* 1970). Since then it has become abundantly clear that patients with HCC, irrespective of their geographical background, consistently have a significantly higher prevalence of markers of current HBV infection than do control subjects (Ohta 1976; Szmuness 1978; Kew 1981; IARC Monographs 1994). The prevalence of HBsAg in patients with HCC is appreciably higher than it is in patients with cirrhosis or chronic hepatitis, providing further support for a specific carcinogenic effect of the virus. In populations with the highest incidences of HCC and in which chronic HBV infection is endemic or hyperendemic, HBsAg can be detected in the serum of as many as 85% of patients with HCC compared with 15% or less of control subjects. Lower prevalences of HBs antigenaemia (usually less than 25%) are found in patients with HCC from countries with a low or an intermediate incidence of the tumour. Even then, the rates are greatly in excess of those in the control subjects, and the relative risk for HCC development is similar to that in high incidence regions. In populations with low or intermediate risk for HCC, risk factors other than HBV, especially persistent HCV

infection and alcoholic cirrhosis, play a greater role in predisposing to HCC.

HBV has also been implicated in the aetiology of HCC in patients who do not have serological evidence of infection with HBV (Kew *et al*. 1986). The correlation between HBV infection and HCC may thus be closer than the above figures indicate. HBV DNA may persist in low concentration in serum even after the disappearance of HBsAg and the appearance of anti-HBs (Zhang *et al*. 1993; Kato *et al*. 1996; Loriot *et al*. 1997). In some patients, HBsAg and antibody to HBsAg (anti-HBs) are absent, while antibody to HBV core antigen (anti-HBc) is detectable in high titre or as IgM anti HBc (Kubo *et al*. 1977; Kew *et al*. 1979; Kew *et al*. 1980). Also, the presence of HBs Ag/anti HBs immune complexes in the serum of patients with HCC may result in the evasion of HBsAg detection by conventional serological tests (Ackerman *et al*. 1994).

Immunodetection techniques and empirical orcein staining have demonstrated HBsAg in nontumorous liver tissue of 32–74% of patients with HCC in countries with a high or intermediate incidence of the tumour (Theodoropoulos *et al*. 1975; Nayak *et al*. 1977; Kew *et al*. 1980). HBsAg was detectable in nontumour tissue in 12–21% of patients from low HCC incidence regions (Peters *et al*. 1977; Nazarewicz *et al*. 1977; Turbitt *et al*. 1977; Trevisan *et al*. 1978). HBsAg is found less frequently (8–22.5%) and in fewer tumour cells (Nayak *et al*. 1977). Also, HBsAg is often detected at the periphery of the tumour and usually has a perinuclear distribution (Nazarewicz *et al*. 1977; Turbitt *et al*. 1977; Trevisan *et al*. 1978; Kew *et al*. 1980). Moreover, the antigen is more readily demonstrable in well or moderately well differentiated malignant hepatocytes. HBV core antigen (HbcAg) has also been detected in nontumorous liver tissue and, less frequently, in the nuclei of malignant cells.

Support for the observation that HBV can be present in malignant hepatocytes was initially provided by a HCC growing in tissue culture (PLC/PRF/5 cell line) that contained integrated HBV DNA and secreted HBsAg into the culture medium (MacNab *et al*. 1976). Other such cell lines have since been reported. In addition, HBV DNA was later shown to be integrated into chromosomal DNA in malignant hepatocytes (Bréchot *et al*. 1981; Shafritz & Kew 1981).

Cohort studies. In those populations with high incidences of HBV-induced HCC, HBV infection is usually acquired perinatally, in infancy or in early childhood (Stevens & Szmuness 1980; Botha *et al*. 1984). In ethnic Chinese populations HBV transmission occurs predominantly as a result of perinatal transmission from highly infectious HBV e antigen (HBeAg) positive carrier mothers. Later horizontal infection plays a secondary role. Conversely, in sub-Saharan Africa, horizontal spread of the virus amongst recently infected and hence highly infectious young children is the predominant route of infection and perinatal transmission is of lesser importance. The exact modes of horizontal spread of infection are not known. Weeping sores or ritual scarification with unsterile instruments may be important. Bites by bloodsucking vectors, such as mosquitoes, bed bugs and hard ticks have been suggested as a possible route, although no proof is yet available.

The difference in the mode of HBV infection in the two populations is attributed to differing frequencies of HBeAg-positivity. In ethnic Chinese women of childbearing age who are chronic carriers of HBV, 40% or more are HBeAg-positive and this is associated with a considerable risk of perinatal infection (Stevens & Szmuness 1980; Beasley & Hwang 1984), whereas in black African women the prevalence is appreciably lower (1–14%) (Botha *et al*. 1984; Kew *et al*. 1987). Nevertheless, black African HBeAg-positive carrier mothers are as likely to transmit the infection perinatally as are ethnic Chinese mothers (Botha *et al*. 1984). Vertical transmission is rare, even with HBeAg-positive mothers.

Children infected with HBV as neonates, infants or very young children have an 80–90% chance of becoming chronic carriers of the virus (Kew *et al*. 1987), and it is these early onset carriers that are at increased risk for HCC. A cohort study from Taiwan estimated that HBV carriers face a lifetime relative risk for developing HCC of more than 100 and approximately 40% of these carriers die from HCC, cirrhosis, or both diseases (Beasley *et al*. 1981). HBeAg-positive carriers and those with cirrhosis are especially prone to tumour formation. In other studies in populations in which infection is predominantly acquired very early in life, relative risks for the development of HCC have ranged from 8.2 to 194 (IARC Monographs 1994). The cohort study performed in Taiwan first suggested the possibility that even those HBV carriers who appear to eliminate the virus remain at increased risk for the development of HCC. A similar observation was reported in a study of woodchucks chronically infected with woodchuck hepatitis virus (WHV), another member of the *Hepadnaviridae* (Korba *et al*. 1989). There are two explanations for the development of HCC in patients who have apparently recovered from chronic HBV infection. HBV DNA may persist in low concentration in the serum and liver tissue after the disappearance of HBsAg from the serum (Zhang *et al*. 1993; Kato *et al*. 1996; Loriot *et al*.

1997). Alternatively, HBV DNA may become integrated into host DNA during an early stage of infection before the elimination of the virions (Shafritz *et al*. 1981; Yu *et al*. 1997).

HBV carriage precedes the appearance of HCC by several or many years, an interval commensurate with a cause-and-effect relationship between the virus and the tumour. The risk for HCC increases progressively with the duration of chronic HBV infection (Ohta 1976; Szmuness 1978). When a tumour presents, the serum titres of HBsAg are usually lower than in carriers without HCC. This suggests that viral replication becomes less active before the tumour supervenes.

Anecdotal evidence indicates that the likelihood of HCC development in individuals infected with HBV in adulthood is considerably lower than that if infection is acquired early in life. A study on United States military personnel who developed acute hepatitis B infection during World War II showed that few subjects progressed to chronic liver disease and even fewer developed HCC (Seeff *et al*. 1987). However, it has been questioned whether follow up of this cohort study was sufficiently prolonged to allow for tumours to develop.

Although HCC is thought to be multifactorial in aetiology, some evidence indicates that HBV can act alone as a risk factor for the tumour (Lohiya *et al*. 1985). HBsAg-positive mentally handicapped children, growing up in an environment thought to be free of aflatoxin B_1 and other known chemical carcinogens, had a relative risk for developing HCC of 246. The malignancy in these patients did not coexist with cirrhosis. Experimental evidence from transgenic mice that express HBV genes (Chisari *et al*. 1989; Kim *et al*. 1991) and woodchucks infected neonatally with WHV and reared in a carcinogen-free environment (Gerin *et al*. 1986) also supports an oncogenic effect of HBV alone.

Final proof of the oncogenicity of HBV may need to await the disappearance of HBV-associated HCC following the eradication of HBV infection by global immunization. Although this outcome will take many years to be realized, there is cause for optimism. In countries in which HBV infection is endemic and immunization has been carried out for a sufficient period, the carrier rate has decreased significantly. In Taiwan, where universal immunization of infants was initiated in 1986, the HBV carrier rate among children has decreased approximately 10-fold (Chang *et al*. 1997) and there has already been a 50% reduction in the incidence and mortality rate from HCC in children.

Indirect evidence for a causal link between HBV and HCC is provided by the observation that other members of the *hepadnaviridae* also induce HCC in their respective hosts. Woodchucks infected with WHV in the neonatal period invariably develop HCC within two years (designating this virus as the most efficient oncogenic agent among recognized hepatocarcinogens (Bréchot 1987)). The corresponding figures in ground squirrels chronically infected with ground squirrel hepatitis virus (GSHV)are approximately 30% after 5–6 years (Marion *et al*. 1987). Integrated sequences of hepadnaviral DNA have been shown to be present in the chromosomal DNA in the tumours in the majority of both of these animal models.

Pathogenesis of hepatitis B virus-related hepatocellular carcinoma

Like all forms of carcinogenesis, HBV-induced hepatocarcinogenesis is a multistep process (Sugimara 1992). Host factors contribute initially to increased proliferation and then to malignant transformation of hepatocytes. Although much remains to be learnt about the pathogenesis of HBV-related HCC, it has become increasingly evident that both direct and indirect processes are involved. Direct mechanisms of hepatocyte transformation include a role for HBV DNA integration, virus mutations, transcriptional activation of growth regulatory genes by HBV-encoded proteins as well as effects on apoptosis, cell signalling and DNA repair.

Direct hepatocarcinogenicity

HBV integration. Integration of HBV DNA into the genome of hepatocytes has been detected in a high proportion of HBV-related HCC (Bréchot *et al*. 1981; Shafritz *et al*. 1981; Takada *et al*. 1990a). This finding has prompted intense investigation of the role of this process in hepatocarcinogenesis. Integration of hepadnavirus DNA into the host genome is not an orderly event, and unlike retroviruses, hepadnavirus integration is not required for viral replication. Hepadnaviruses do not encode an integrase enzyme and integration occurs during a recombination event that requires host cellular enzymes.

Intact and complete HBV genomic DNA has not been demonstrated in any of the integrants in HBV-infected hepatocytes (Dejean *et al*. 1984; Koch *et al*. 1984; Mizusawa *et al*. 1985; Yaginuma *et al*. 1985; Ziemer *et al*. 1985; Hino *et al*. 1986; Nagaya *et al*. 1987; Shih *et al*. 1987; Robinson 1994). Deletion of some viral sequences has usually been found and no two described HBV integrants are the same. The cellular DNA mutations resulting from HBV integration are also

highly varied. They include small and large deletions, chromosomal translocations, and head to tail duplication of viral and cellular elements as well as amplification of sequences. HBV integration at cellular genes thought to be important in the regulation of cell division have rarely been described (see below). The preferred sites of HBV DNA integration appear to be repeat elements such as Alu and minisatellite sequences. Although some general features of HBV integration in HCC have emerged, identification of the molecular basis of the link between HBV DNA integration and hepatocarcinogenesis has been complicated.

Mechanisms of HBV DNA integration. Most cases of HBV integration have been characterized in advanced stages of chronic infection in which HCC is also present (Bréchot *et al*. 1981; Koshy *et al*. 1981; Shafritz *et al*. 1981; Hada *et al*. 1986; Yaginuma *et al*. 1987; Takada *et al*. 1990). Viral DNA integration in the initial phases of carcinogenesis is less well described. Insertion of HBV DNA may occur early during persistent infection and selective clonal amplification of hepatocytes, with their own unique integration patterns, is thought to occur during progression to malignancy (Rogler & Summers 1984; Rogler & Chisari 1992).

The detailed structure of several hepadnavirus DNA integrants has been defined by sequencing virus-virus and virus-cell junctions (Dejean *et al*. 1984; Koch *et al*. 1984; Mizusawa *et al*. 1985; Yaginuma *et al*. 1985; Ziemer *et al*. 1985; Hino *et al*. 1986; Nagaya *et al*. 1987; Shih *et al*. 1987). HBV DNA integrants have been detected at single or multiple sites of cellular DNA. In most HBV-related HCCs, hepadnavirus DNA sequences are integrated at three or four different cellular sites. HBV DNA may insert as a single linear sequence although it usually comprises contiguous fragments of rearranged viral sequences. Virus-virus junctions may be joined in head to head, or head to tail arrangements.

Sites on the HBV genome that are located more commonly at virus-host junctions have been identified. Integrations that occur at or near the cohesive overlap region of the partly double stranded HBV genome between the direct repeat sequences (DR1 and DR2), account for two thirds of the sequenced integrants (Dejean *et al*. 1984; Koch *et al*. 1984; Mizusawa *et al*. 1985; Yaginuma *et al*. 1985; Ziemer *et al*. 1985; Hino *et al*. 1986; Nagaya *et al*. 1987; Shih *et al*. 1987; Robinson 1994). The patterns of HBV DNA insertion have thus been categorized (I, II, III and IV) according to an idealized structure of the viral sequences located at the virus-host integration sites (Figure 2). Among the cohesive region integrants, virus-cell junctions that are clustered around DR1 (patterns I and II) are found three times more commonly than around the DR2 sequence (patterns III and IV). Of the junctions around DR1, pattern II is commonest and accounts for 46.6% of the cohesive overlap integrants in HCC. The region between DR1 and DR2 is also the preferred site for the formation of virus-virus junctions. Other recombination-proficient regions of HBV have been located in the preS/S region of HBV. These sites correspond with the transcription initiation sites of the subgenomic HBV mRNA species and reverse transcription of this mRNA may contribute to the mechanism of integration at these sites.

DR1 and DR2 are required to initiate minus and plus strand HBV DNA synthesis as well as for template strand switching during reverse transcription of pregenomic HBV DNA. The clustering of virus-virus and virus-cell HBV integrants around the DR1 and DR2 sequences suggests that intermediates of viral replication serve as the substrates for viral integration (Nagaya *et al*. 1987; Shih *et al*. 1987). Strand invasion of host cellular DNA by single stranded linear HBV DNA is thought to initiate the recombination. Clustering of the integration sites around the cohesive end region suggests that the 5′ ends of single stranded HBV DNA

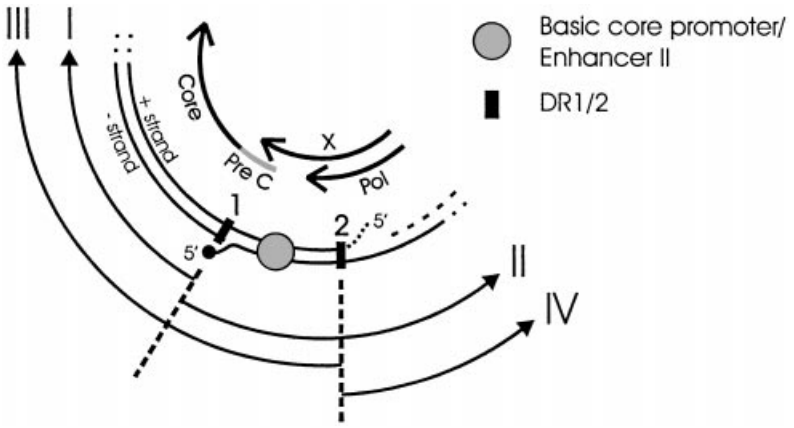

Figure 2. Cohesive overlap and surrounding regions of hepatitis B virion DNA with commonly observed HBV DNA integration patterns. The viral plus DNA strand with RNA attached at the 5′ end (dotted line) and minus DNA strand with covalently attached terminal protein (solid circle) are indicated. DR1/2, overlapping cis-elements (basic core promoter/enhancer II) and open reading frames (polymerase, X and preC/Core) are also shown. I, II, III and IV represent idealized patterns of commonly observed HBV DNA integrants in cellular DNA of HBV-related HCC.

are responsible for the strand invasion of cellular DNA. Integration pattern II is the most frequently observed, indicating that minus strand DNA invasion is the most common. Invasion of the 5′ end of plus strand DNA (pattern III) occurs less frequently. Recombination patterns I and IV may result from invasion from the 3′ ends of minus and plus strands, respectively. Homology between viral and cellular DNA (Yaginuma *et al*. 1985), as well as repeats of sequences that flank the viral insert (Koch *et al*. 1984), have been described and may play a part in the mechanism of integration in some cases. Data from a recent study suggested that interaction of the Yin and Yan1 transcription initiation factor (YY1) is involved in the formation of virus-cell junctions during recombination of viral and cellular DNA (Nakanishi-Matsui *et al*. 2000). However, the details of this mechanism remain to be established.

An attractive explanation for HBV integration, involving cleavage of cellular and partially double stranded HBV DNA by topoisomerase I, has been proposed (Wang & Rogler 1991). Topoisomerase I is an abundant cellular enzyme that relieves superhelical tension of DNA during DNA replication and transcription by introducing single strand breaks into duplex DNA (Wang 1985). The sequence located in the hepadnavirus cohesive overlap is a preferred topoisomerase I cleavage substrate (Wang & Rogler 1991). Topoisomerase I-mediated single strand cleavage in the cohesive overlap is thought to destabilize the circular structure of partially double stranded HBV DNA, and to generate linearized HBV DNA that would be available for rejoining to heterologous cellular DNA. Also, topoisomerase I cleavage of HBV DNA results in the formation of single stranded protruding DNA sequences that are sensitive to cellular nucleases. This would explain the common observation of microdeletions in HBV integrants. *In vitro*, it was demonstrated that topoisomerase I is capable of mediating recombination of WHV DNA with cellular DNA. Moreover, the integration *in vitro* was similar to hepadnavirus integrations that have been described in HCCs.

Cis-activation of cellular genes by hepadnavirus integration. Mutation of cellular growth regulatory genes by insertion of hepadnavirus DNA as a putative mechanism of hepatocarcinogenesis has led to several investigations aimed at characterizing the alterations of cellular genes that occur at integration sites. Integration of hepadnavirus DNA may disrupt host cellular sequences by changing a host protein-encoding sequence and also by insertion of viral regulatory sequences that alter the normally tightly controlled growth regulatory genes. Sequences in the region of common hepadnavirus integration sites that may be disruptive to normal cellular gene transcription include the basic core promoter/enhancer II, the X promoter and *HBx* sequence. However, HBV integration into host DNA that may induce cellular transformation by this mechanism seems to be a rare event (Robinson 1993; Robinson 1994). Interestingly, this contrasts significantly with the hepatocarcinogenic mechanism that occurs in liver cancer asociated with WHV infection. WHV integrants have been observed at or near the c-*myc* and N-*myc* proto-oncogenes in 50% of WHV-associated HCC (Hsu *et al*. 1988; Etiemble *et al*. 1989; Moroy *et al*. 1989; Fourel *et al*. 1990; Hansen *et al*. 1993). The sites of WHV integration within the *myc* genes have varied. Integrants are usually located upstream or downstream of Myc-encoding sequences and fusions of WHV and *myc* open reading frames are an unusual observation. In ground squirrels, c-*myc* overexpression and amplification is frequently observed in GSHV-associated liver cancer. However, the mechanism of *myc* activation does not appear to involve GSHV DNA integration at or near c-*myc* (Transy *et al*. 1992).

There have been few examples of HBV integration at cellular sequences that are potentially oncogenic. In one study, a single integration site was defined within the second intron of the cyclin A gene (Wang *et al*. 1990). Cyclins, cyclin dependendent kinases and cyclin dependent kinase inhibitors interact to play an important role in regulating cell division. In the HBV-cyclin A mutant protein, the first 152 amino-terminal amino acids of cyclin A were replaced by 156 amino acids of the preS2/S middle surface protein of HBV. The HBV-cyclin A fusion was shown to have oncogenic properties which are lacking in either the viral sequence or amino-terminally deleted cyclin A proteins alone (Wang *et al*. 1992; Berasain *et al*. 1998).

Potentially hepatocarcinogenic HBV integration at the retinoic acid receptor (RAR) β gene has also been described (Dejean *et al*. 1986). RARs belong to a family of ligand-regulated transcription factors that are important for the control of cell differentiation and proliferation. In a HBV-related HCC, the RAR β gene was mutated by insertion of a continuous HBV sequence that included the cohesive overlap from DR2, the core gene and preS1 sequences (pattern III, Figure 2). The viral-host fusion protein comprised the first 29 codons of preS1 in frame and upstream of the DNA binding domain of RAR β. The HBV integrant included the preS1 promoter and the resulting preS1-RAR β fusion protein was transcribed from the preS1 promoter. Assessment of the oncogenic properties of RAR β and the HBV-RAR β

fusion using avian erythroblastosis-based retroviral vectors demonstrated that fusion of the HBV preS1 sequence to the RAR β gene resulted in overexpression of a mutant protein and enhanced carcinogenic properties of RAR β (Garcia *et al*. 1993).

Other examples of HBV integration at cellular sequences that are important for regulating cell proliferation have been described. An HBV integrant was located within a sequence that shows a high degree of similarity to the tyrosine protein kinase domain of the human epidermal growth factor receptor gene (Zhang *et al*. 1992). Integration of HBV DNA at the mevalonate kinase gene in the PLC/PRF/5 hepatoma cell line (Graef *et al*. 1994; Graef *et al*. 1995) and the carboxypeptidase gene (Pineau *et al*. 1996) in HBV-associated HCC has also been described. The significance of these integration events, however, remains to be clarified.

Transcriptional activation by HBV-encoded proteins

The genome of hepadnaviruses has a similar organization to that of retroviruses and areas of significant sequence homology have been identified (Miller & Robinson 1986). This observation, together with the fact that replication of both groups of virus involves reverse transcriptase, suggests that retroviruses and hepadnaviruses have common ancestral origins. The X ORF, encoding the HBx protein, is located at the 3′ end of the linearized pregenomic RNA in a position analagous to the location of sequences that encode accessory transcriptional regulating proteins of complex retroviruses (e.g. Tat protein of HIV-1). This prompted investigation of a role for HBx in transcriptional regulation, a role subsequently confirmed. HBx is the smallest HBV protein and is required for WHV infection (Chen *et al*. 1993; Zoulim *et al*. 1994) and presumably for the replication of HBV *in vivo*. The X ORF is conserved in mammalian hepadnaviruses associated with HCC. Oncogenic effects of avian hepadnaviruses, which are devoid of the X ORF, have not been demonstrated. It is unclear whether *trans*-activation is the essential HBx function required for HBV replication *in vivo*. Evidence that it is comes from the observation that HBx is required to activate transcription of the core gene *in vivo* in transgenic mice (Reifenberg *et al*. 1999). Activation of the expression of cellular growth regulatory genes during processes that are potentialy oncogenic has been an area of major interest in evaluating the *trans*-activator function of HBx.

A consequence of HBV integration in the cohesive overlap region, and in particular the high incidence of pattern II type integrations, is that *HBx* sequences are often found in the integrants. Although *HBx* transcripts are frequently detected in HBV-associated HCC (Paterlini *et al*. 1995), the integrants are often rearranged. Changes to the *HBx* ORF include truncation with fusion to cellular DNA. An intact *HBx* sequence is an unusual finding in HBV integrants and has been estimated to be present in considerably fewer than 50% of HBV-related HCC. However integrated *HBx*, even when truncated, often encodes functionally active *trans*-activator proteins and may overexpress these proteins (Schluter *et al*. 1994). Characterization of HBx expression in malignant hepatocytes and infected liver tissue has been complicated by the difficulty in obtaining high-affinity antibodies against HBx for immunodetection. A recent study indicated that HBx expression occurs in a small number of malignant cells and in preneoplastic hepatocytes (Su *et al*. 1998). Several studies on HBx transgenic mice have investigated the hepatocarcinogenic effects of integrated *HBx*. Initial reports showed that mice harboring HBx develop progressive features that are characteristic of multistep malignant transformation of liver cells (Kim *et al*. 1991; Ullrich *et al*. 1994). Not all HBx transgenic mice develop HCC and some reports indicate that expression of HBx above a certain threshold is necessary for transformation of hepatocytes (Koike 1995; Koike *et al*. 1994). Other lineages of HBx transgenic mice do not develop liver pathology unless exposed to additional hepatocarcinogenic influences such as exposure to diethylnitrosamine (a carcinogen) (Billet *et al*. 1995; Slagle *et al*. 1996; Terradillos *et al*. 1997).

The preS1/preS2/S sequence, encoding the large and middle hepatitis B virus surface proteins (LHBs and MHBs, respectively), is another region of HBV that encodes a transcriptional activator with potentially transforming properties (reviewed in Caselmann 1996; Hildt & Hofschneider 1998). Interestingly, *trans*-activating properties are only gained after carboxy-terminal truncation of LHBs or MHBs. Support for a role for the large surface protein in hepatocarcinogenesis comes from the observation that transgenic mice expressing this protein develop HCC (Chisari *et al*. 1987). As with HBx, truncated MHBs ($MHBs^t$) has pleiotropic effects on gene transcription. The *trans*-activating effects are mediated by modulating protein kinase C signal transduction, interaction with several transcription factors as well as by sequence-specific binding to DNA (Kekule *et al*. 1990; Hildt *et al*. 1996; Alka *et al*. 2000). Potentially oncogenic transcriptional effects of $MHBs^t$ include the stimulation of promoter sequences of the c-*myc*, c-*fos* and c-Ha-*ras* oncogenes (Meyer *et al*. 1992; Natoli *et al*. 1992; Lauer *et al*. 1994).

Mechanisms of transcriptional activation by HBx. The mechanism of transcriptional activation by HBx has been the subject of several investigations. HBx activates gene expression by increasing the rate of transcription, and not by stabilizing mRNA as do some of the retroviral accessory proteins (e.g. Tat protein of HIV1) (reviewed in Rossner 1992; Caselmann 1996; Yen 1996; Murakami 1999; Arbuthnot *et al*. 2000). Direct binding of DNA by HBx has not been described and it effects transcriptional activation by intracytoplasmic modulation of signalling pathways and interaction with nuclear transcription factors. The nuclear and cytoplasmic location of HBx in transfected and infected cells is consistent with transcriptional activation that involves these two mechanisms. The cell signalling pathways activated by HBx include mitogen activated protein kinase (MAPK) (Kekule *et al*. 1993; Luber *et al*. 1993; Benn & Schneider 1994; Benn *et al*. 1996) and Janus family tyrosine kinase (JAK)/signal transducer and activators of transcription (STAT) pathways (Lee & Yun 1998) (see below). Proteins that are involved in controlling expression of specific genes as well as general transcription factors interact with HBx. The wide-ranging effects of HBx on components that regulate gene transcription account for the pleiotropic nature of HBx *trans*-activation. Most studies have shown that the amino-terminal third of HBx can be removed without affecting transcriptional activation (Ritter *et al*. 1991; Arii *et al*. 1992; Renner *et al*. 1995). The amino-terminal domain includes an element that acts as a negative regulator of HBx *trans*-activation and which is also important for HBx dimerization (Murakami *et al*. 1994). The regions that are thought to be important for transcriptional activation are located from amino acids 50–70 as well as from 110 to 140 (Koike *et al*. 1989; Caselmann *et al*. 1990; Levrero *et al*. 1990; Unger & Shaul 1990; Balsano *et al*. 1991; Ritter *et al*. 1991; Yen 1996).

There are several *cis*-elements that are responsive to transcriptional activation by HBx (reviewed in Rossner 1992; Caselmann 1996; Yen 1996; Murakami 1999). These include sequences from HBV, heterologous viruses and cellular transcriptional regulatory elements. Enhancer I in the HBV genome is responsive to *trans*-activation by HBx (Twu & Schloemer 1987; Spandau & Lee 1988; Colgrove *et al*. 1989; Siddiqui *et al*. 1989; Faktor & Shaul 1990). This effect may be required for efficient expression of HBV genes and synthesis of HBV pregenomic RNA. Heterologous viral *cis*-elements that are stimulated by HBx include the SV40 early promoter/enhancer (Twu & Schloemer 1987; Spandau & Lee 1988) and HIV1 LTR (Siddiqui *et al*. 1989; Twu & Robinson 1989). Many cellular targets are activated by HBx. Genes that are activated include the cellular oncogenes c-*myc* (Balsano *et al*. 1991), c-*fos* (Avantaggiati *et al*. 1993) and c-*jun* (Twu *et al*. 1993), β-interferon (Twu & Schloemer 1987) and $tRNA^{Ala}$ (Aufiero & Schneider 1990). The activation of gene expression may thus be important for viral infection and also for the disruption of cellular genes that normally regulate hepatocyte proliferation.

Interaction of HBx with specific transcription factors. Mobility shift assays showed that HBx binds to CREB and to ATF-2 transcription factors (Maguire *et al*. 1991). CREB and ATF-2 mediate transcriptional responses to cyclic AMP (cAMP) by binding to the cAMP responsive element (CRE, 5′ TGACGTCA 3′) (Johnson & McKnight 1989; Ziff 1990). CREB is a leucine zipper protein that binds to CRE as a homodimer. A CRE-like sequence within HBV enhancer I (5′ TGACGCAA 3′) is incapable of binding CREB and ATF-2 alone (Maguire *et al*. 1991). However, HBx interacts with the DNA binding domain of CREB to effect increased transcriptional activation by CREB through the formation of CREB or ATF-2 complexes with the HBV CRE-like element (Williams & Andrisani 1995).

Further investigations revealed that HBx not only interacts with CREB, but also with other members of the bZip group of dimerizing leucine zipper transcriptional factors. These factors are involved in mediating stress responses and in the regulation of the acute phase inflammatory response (Barnabas *et al*. 1997). bZip proteins include ATF3, ICER IIγ, gadd153/Chop10 and NF-IL6, which may activate or repress gene expression in the liver. HBx enhances both transcriptional activating and repressive efficacy of bZip factors (Barnabas *et al*. 1997; Barnabas & Andrisani 2000). An inhibitory effect of HBx on transcription of the human homologue of the yeast *sui1* gene (hu-*sui1*), which encodes a translation initiation factor has also been reported recently (Lian *et al*. 1999). *Trans*-repressive effects of HBx may contribute to hepatocarcinogenesis by inhibiting negative growth regulatory effects.

HBx also increases the DNA binding and activation properties of the transcription factors NF-κB and AP-1 (Lucito & Schneider 1992; Cross *et al*. 1993; Benn & Schneider 1994; Natoli *et al*. 1994a; Su & Schneider 1996). Cytoplasmic activation of Ras-Raf-MAPK pathways is responsible for this effect of HBx (Doria *et al*. 1995). NF-κB modulates cellular stress responses and has been shown to control the expression of several acute phase response proteins, cytokines and adhesion molecules (reviewed by Gosh *et al*. 1998). NF-κB is a heterodimer protein comprising p50 or p52 and RelA

subunits (Gosh *et al*. 1998). The Rel-homology domain (RHD) is common to the Rel family of transcription factors and confers DNA binding and nuclear localization properties on NF-κB. IκB-α, an NF-κB inhibitor, sequesters NF-κB in the cytoplasm and renders it inactive. Phosphorylation of amino-terminal serine residues of IκB-α leads to its polyubiquitination and subsequent degradation in proteasomes. During this process, NF-κB is released and translocates to the nucleus where it exerts effects on gene expression. The enzymes that ubiquitinate IκB are constitutively active and phosphorylation of IκB is the important regulatory step (Karin & Delhase 1998; Karin 1999). Phosphorylation of IκB-α is stimulated by HBx and leads to the diminished stability of IκB-α and activation of NF-κB (Su & Schneider 1996) (Fig. 3). IκB kinase (IKK) is the enzyme complex that responds to NF-κB inducing stimuli by phosphorylation of IκB serine residues. In turn, phosphorylation activates IKK by an incompletely characterized pathway. The mitogen activated protein kinase kinase kinase (MAPKKK) NIK acts as a powerful IKK and NF-κB activator. Stimulation of Ras-Raf-MAPK pathways by HBx is well described (see below) and may be the mechanism of NF-κB activation by HBx (Benn *et al*. 1996).

The induction of NF-κB is a rapid reaction that does not require *de novo* protein synthesis. IκB-α is replenished by a feedback mechanism involving NF-κB-mediated activation of the IκB-α gene. Other inhibitors of NF-κB include the NF-κB precursors, p105 and p100, which have IκB-α-like regions in their carboxy terminal domains. During their maturation, p105 and p100 are modified by proteolytic removal of the carboxy-terminals responsible for cytoplasmic sequestration of NF-κB. HBx also induces a decrease in the cytoplasmic concentration of p105 precursor protein, which in turn leads to activation of NF-κB by a different pathway (Su & Schneider 1996). This action is similar to the effect of the HTLV-I Tax protein on NF-κB function (Kanno *et al*. 1994).

A role for HBx in modulating proteasome function may also be important in regulating the concentration of

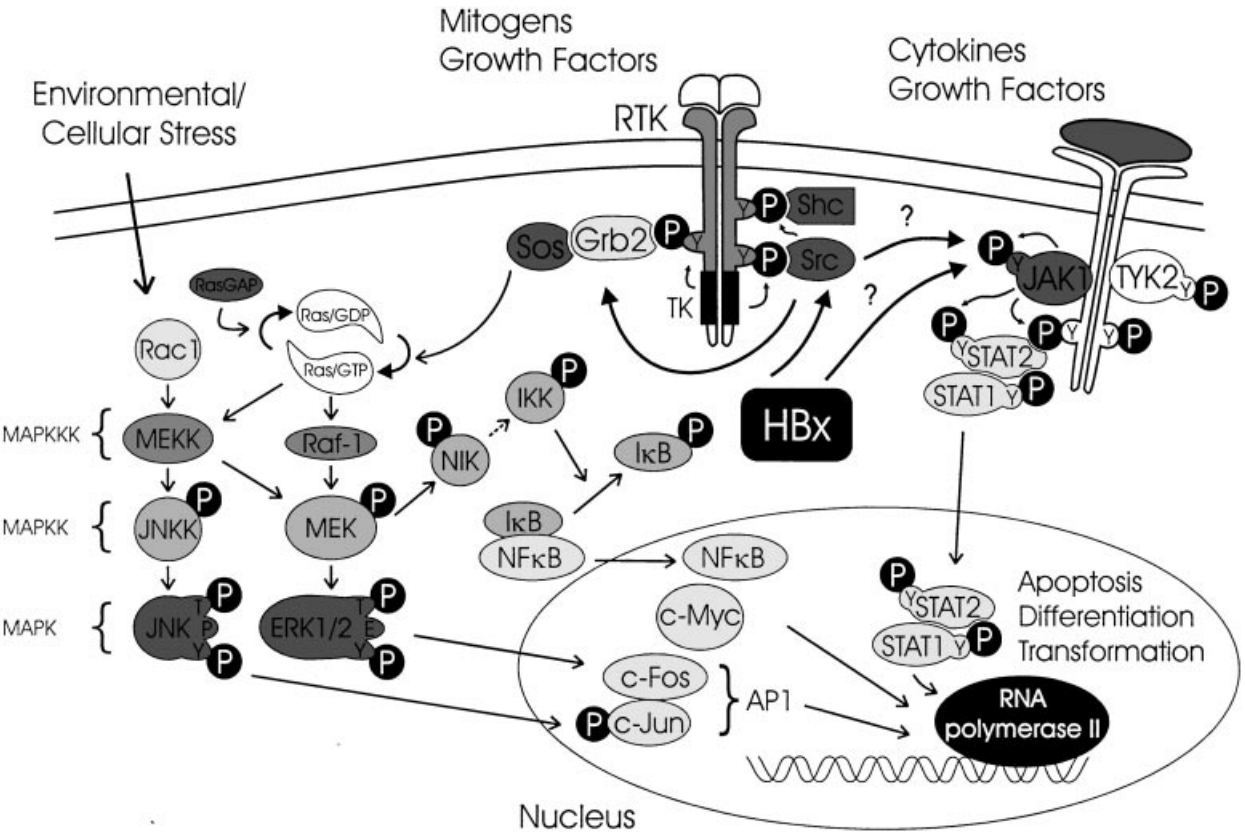

Figure 3. HBx activation of NF-κB and MAP kinase and JAK/STAT cellular signalling pathways. Arrows indicate the major stimulatory routes of signal transduction. As examples of the mitogen activated protein kinase (MAPK) pathways, Ras/Raf/MEK/MAPK and Jun N-terminal kinase cascades are indicated. RTK, receptor tyrosine kinase; TK, intracellular tyrosine kinase; P, phosphate; Y, tyrosine; T, threonine; S, serine. See text and related references for further details.

NF-κB. Observations supporting the idea that HBx interacts with the proteasome machinery include the colocalization of HBx with proteasomes in the cytoplasm of transfected cells, inhibition of the degradation of proteins by the ubiquitin-proteasome pathway and HBx interaction with PSMA7 and PSMC1 proteasome subunits (Sirma *et al*. 1998; Hu *et al*. 1999; Zhang *et al*. 2000). PSMC1 is a member of a family of ATPases that is linked to the functioning of several transcription factors. Proteins whose degradation may be inhibited by HBx include a general transcription factor (Zhang *et al*. 2000) and p105 (Sirma *et al*. 1998). However, this observation may be preliminary as it conflicts with the previously reported diminished p105 stability effected by HBx, and which contributes to the well-characterized activation of NF-κB by HBx (Su & Schneider 1996).

HBx also causes activation of the transcription factor AP-1 (Lucito & Schneider 1992; Cross *et al*. 1993; Benn & Schneider 1994), although the rate of cell division may modulate this HBx-mediated effect (Henkler *et al*. 1998). AP-1 is a dimer that comprises Fos and Jun leucine zipper proteins (Curran & Franza 1988). Activation of Ras-Raf-MAPK cascades stimulate c-*fos* and c-*jun* expression to increase intracellular Fos and Jun protein concentrations (Davis 1993; Denhardt 1996). The extracellular signal regulated kinases (ERKs) activate c-*fos* expression, and Jun N-terminal kinases (JNKs) are reponsible for c-*jun* activation. Jun is activated by multisite phosphorylation within the N-terminal domain, which in turn leads to the autostimulation of c-*jun* expression (Fig. 3). Unlike the effect on NF-κB, HBx increases *de novo* synthesis of Jun and Fos and stimulates AP-1 *trans*-activation by activating both ERK and JNK MAPK pathways (Natoli *et al*. 1994a; Natoli *et al*. 1994b; Benn *et al*. 1996). Results from cotransfection of dominant negative mutants confirm that HBx acts on each of these independently regulated pathways. HBx increases expression of Fos transiently and results in short-lived accumulation of Fos-Jun AP-1 heterodimers. However, HBx stimulation of Jun expression is sustained and leads to the continued accumulation of Jun-Jun AP-1 homodimers (Benn *et al*. 1996).

Studies have also demonstrated that HBx interacts with general transcription factors that are required for the transcription of most cellular genes. Investigations using bacteriophage library screening and immunoprecipitation showed that HBx binds the fifth subunit of RNA polymerases (RPB5) *in vivo* and *in vitro*, and the association of the two proteins mediates transcriptional activation (Cheong *et al*. 1995). Interestingly, the same study showed that the association of RPB5 with HBx did not activate transcriptional regulatory sequences that lacked X-responsive elements. The reason for this selective effect of HBx on a general transcription factor is not clear. Interaction of HBx with other general transcription factors, TFIIB (Haviv *et al*. 1998) and components of TFIIH (Wang *et al*. 1994; Quadri *et al*. 1996), has also been demonstrated, although binding between HBx and TFIIB has not been a constant observation. Quadri *et al*. (1996) reported that a recombinant HBx fusion protein (GST-HBx) binds to TATA binding protein (TBP) but not to TFIIB. The interaction between HBx and TBP may be important for *trans*-activation, while binding between HBx and TFIIH may regulate both transcription as well as DNA repair (see below).

Recently, a protein that has negative regulatory effects on HBx *trans*-activation has been isolated (Melegari *et al*. 1998). The protein, termed XIP for HBx-interacting protein, was shown to bind to the carboxy-terminal region of HBx. XIP abolished *trans*-activation by HBx and inhibited HBV replication in transfected cells. The precise physiological role of this protein remains to be established.

HBx activation of the MAP kinase and JAK/STAT pathways

The JAK/STAT and MAP kinase pathways modulate transcriptional activity of a wide range of transcriptional factors that are involved in regulating cellular growth, differentiation and programmed cell death (apoptosis) (Davis 1993; Denhardt 1996). Signalling via these pathways is a complex system involving cross-talk, feedback loops and multicomponent complexes. Stimulation of the MAPK cascades by Ras/GTP typically involves the sequential activation of three groups of protein kinases (Figure 3). A serine/threonine protein kinase (MAP kinase kinase kinase or MAPKKK) phosphorylates and activates a dual specificity protein kinase (MAP kinase kinase or MAPKK). Phosphorylation of threonine and tyrosine residues of MAP kinase (MAPK) by MAPKK in turn activates MAPK. MAPK is the effector of the MAPK pathway and itself acts as a serine/threonine protein kinase. The dephosphorylation reactions are thought to be important for terminating signal transduction pathways but are not well characterized.

Several reports have now demonstrated HBx activation of the Ras/Raf/MEK/ERK and JNK pathways (Figure 3) (Cross *et al*. 1993; Benn & Schneider 1994; Natoli *et al*. 1994a; Benn *et al*. 1996). Some have reported that diacylglycerol-dependent protein kinase C activation mediates HBx *trans*-activation (Kekule *et al*. 1993; Luber *et al*. 1993), but this has not been the finding of most studies (Lucito & Schneider 1992; Cross

et al. 1993; Murakami *et al*. 1994; Natoli *et al*. 1994a). MAP kinase pathway activation has been shown to be required for AP1 and NFκB transcriptional factor activation by HBx (Cross *et al*. 1993; Benn *et al*. 1994; Natoli *et al*. 1994a; Benn *et al*. 1996) (discussed above). HBx increases GTP uptake onto Ras (Klein & Schneider 1997) and stimulates Ras-Raf-MAPK pathways moderately over a prolonged period of time. The activation of Src kinases is likely to be the mechanism underlying HBx stimulation of MAPK cascades (Figure 3). A potentially oncogenic consequence of activation of Src and the Ras/Ras/MEK/ERK pathway by HBx is the resulting increased cell proliferation and deregulation of cell cycle check point controls (Benn & Schneider 1995).

HBx also mediates activation of JAK/STAT signalling pathways (Lee & Yun 1998). The cascade involves activation of cell surface receptors, then JAK protein phosphorylation of itself, as well as receptor components, to create docking sites for STATs (Figure 3) (Pellegrini & Dusanter-Fourt 1997; Leonard & O'Shea 1998). The STAT proteins are themselves activated by phosphorylation of a critical conserved tyrosine residue. After phosphorylation, STATs form stable homo- or heterodimers that rapidly translocate to the nucleus to bind target regulatory DNA elements. STAT binding sites are often in close proximity to motifs for other transcriptional factors that are activated by HBx, and JAK/STAT activation is thought to mediate HBx *trans*-activation in part. HBx stimulates JAK1 tyrosine kinase, which results in the constitutive phosphorylation of various STATs and a concommitant increase in STAT DNA binding and transcriptional activation (Lee & Yun 1998). A possible mechanism of increased signal transmission via JAK/STAT pathways caused by HBx is the stimulation of cell proliferation. Activation of JAK/STAT signalling in the liver has been associated with increased hepatocyte proliferation in response to stimulation by growth factors or partial hepatectomy (Cressman *et al*. 1995; Ruff-Jamison *et al*. 1995). Alternatively, HBx activation of JAK/STAT pathways may be mediated by activation of Src family kinases (Klein & Schneider 1997). This may be a process that is common to stimulation of both MAP kinase and JAK/STAT pathways. The role of the interaction of HBx with Src PTKs remains to be elucidated fully. By modulating Src PTKs, HBx action may have wide-ranging effects on potentially transforming cellular processes and may explain the promiscuous nature of HBx actions.

The effects of HBx on apoptosis

Several proteins encoded by DNA viruses are capable of binding to p53 to activate or inactivate apoptosis (Linzer & Levine 1979; Sarnow *et al*. 1982; Werness *et al*. 1990). Inhibition of apoptosis may enable viruses to replicate by countering cellular apoptotic defence mechanisms. Conversely, a pro-apoptotic effect may facilitate viral spread and allow evasion of host cell-mediated immunity (Debbas & White 1993; Lowe & Ruley 1993). Histological features of apoptosis, for example the presence of Councilman bodies, are commonly seen in HBV-infected livers (Lau *et al*. 1998). Whether or not endogenous cellular apoptotic pathways are modulated during HBV infection of hepatocytes, and in particular the role of HBx in such a process, has been extensively investigated.

Initial reports demonstrated an inhibitory effect of HBx on apoptosis in microinjected human fibroblasts (Wang *et al*. 1995a; Elmore *et al*. 1997). The distal C-terminal region of HBx is required to cause this effect and it is thought to be mediated by inactivation of p53. Also, expression of HBx in rat fibroblasts and hepatoma cells led to cellular resistance to a range of apoptotic stimuli (e.g. Fas antibodies and growth factor depletion) that engage caspase 3 as a downstream effector (Gottlob *et al*. 1998). HBx acted as effectively as competitive caspase inhibitors but did not bind caspase 3 or influence the conversion of the precursor caspase 3 to its active form. The regions required for the inhibition of caspase 3 activity corresponded to the known Kunitz-type domains (Takada & Koike 1990). An investigation using a yeast two-hybrid assay identified a mitochondrial voltage dependent anion channel protein (HVDAC3) as an HBx-interacting protein (Rahmani *et al*. 1998). The colocalization of HBx and HVDAC3 was confirmed in living cells and HBx was shown to decrease the mitochondrial transmembrane potential (Rahmani *et al*. 2000). The possible role of HBx in modulating mitochondrial function during HBV-induced liver injury and HCC remains to be demonstrated. This is especially interesting in the context of the central role of mitochondrial factors (e.g. cytochrome c) in modulating apoptosis.

HBx has been reported to exert a spontaneous pro-apoptotic effect on primary cultures of hepatocytes and in the livers of *HBx* transgenic mice (Koike *et al*. 1998; Pollicino *et al*. 1998; Terradillos *et al*. 1998). These similar observations *in vivo* and in cell culture suggest that HBx effects are not entirely the result of a cytotoxic immune response and that HBx regulates endogenous cellular pathways that control apoptosis. Apoptosis was also enhanced in HBx transgenic mice carrying the p53 null mutation suggesting that p53 is not required to activate HBx mediated apoptosis signal in this model (Terradillos *et al*. 1998). A separate study confirmed

increased hepatocyte apoptosis in HBx transgenic mice, but the emergence of HCC was preceded by a decrease in apoptosis (Koike *et al*. 1998). The formation of transformed foci of NIH3T3 cells transfected with a variety of oncogenes was completely inhibited by HBx. The effect was reversed by the apoptosis inhibitor, Bcl-2, and suggests that HBx induces apoptosis to inhibit the emergence of transformed foci (Kim *et al*. 1998). HBx was also shown to sensitize cultured human hepatocytes and rat fibroblasts to programmed cell death induced by tumour necrosis factor α (TNFα) (Su & Schneider 1997), although HBx-mediated resistance to apoptotic effects of HBx have also been observed (Gottlob *et al*. 1998). TNFα modulates hepatic inflammation and immune-mediated apoptosis in chronic HBV infection. TNFα production is elevated in HBV chronic carriers and the expression of TNFα receptors in infected hepatocytes correlates with hepatic inflammation (Sheron *et al*. 1991). Sensitization of liver cells to TNFα by HBx may be mediated by a prolonged stimulation of *N-myc* transcription and the stress-mediated mitogen-activated-protein-kinase 1 pathway (Su & Schneider 1997). Sensitization of cells to apoptosis caused by TNFα is, however, not a constant observation.

The apparently discrepant effects of HBx on apoptosis may reflect opposing concentration-dependent effects at different stages of natural HBV infection. It is possible that HBx inhibits apoptosis early during hepatocyte infection and later activates apoptosis to facilitate HBV replication and spread. Either stimulation or inhibition of apoptosis could lead to malignant transformation of hepatocytes. Inhibition of apoptosis would allow the accumulation of potentially tranforming mutations. Enhanced hepatocyte proliferation as a compensatory response to pro-apoptotic effects may lead to the selection of premalignant liver cells. *Trans*-activation of growth regulatory genes by HBx would exacerbate this effect. A clear understanding of the role of HBx in the HBV life cycle will undoubtedly assist in identifying the biologically important influences of this protein on apoptosis.

Interaction between HBx and p53. Inhibition of the function of the tumour suppressor protein, p53, has been implicated in the mechanism of malignant transformation in several tumours. Initial investigations on the interaction between HBx and p53 demonstrated that the two proteins associate with each other both *in vivo* and *in vitro* (Feitelson *et al.* 1993). In transgenic mice expressing HBx from integrated HBV DNA, it was shown that tumour development correlates with binding of p53 by HBx (Ueda *et al.* 1995). It was proposed that decreased nuclear concentration of p53, caused by HBx sequestration of p53 in the cytoplasm, inhibits hepatocyte apoptosis. However, cytoplasmic sequestration of p53 has not been consistently associated with inhibition of apoptosis caused by HBx (Wang *et al.* 1995a). In a study on HCC tissue specimens from Chinese patients, immunodetection methods failed to reveal HBx in the tumours and there was no evidence of HBx associated with immunoprecipitated p53 (Henkler *et al.* 1995). However, sequence analysis revealed potentially inactivating p53 mutations in almost all of the tumours. It was suggested that functional inactivation of p53 by HBx may occur early during hepatocarcinogenesis and the predominant nuclear localization of p53 that is observed in HCC (Wang *et al.* 1994; Schaff *et al.* 1995; Okuda *et al.* 1996) may be a late phenomenon that reflects accumulation of mutant and inactive p53.

Several studies have demonstrated effects of HBx on the *trans*-activation properties of p53. HBx was reported to inhibit sequence-specific DNA binding and transcriptional activation by p53 (Wang *et al*. 1994). An interesting converse observation is that p53 inhibits the transcription of *HBx* mRNA from the minimal X promoter (Takada *et al*. 1995). HBx alters the formation of the p53 preinitiation transcription complex but does not inhibit the binding of *cis*-elements by p53 (Truant *et al*. 1995). Important, and potentially carcinogenic consequences of inhibition of p53 transcription may be a failure to transcribe genes involved in cell cycle arrest and pro-apoptotic pathways.

Modulation of DNA repair by HBx

A number of cellular DNA repair pathways exists to maintain genome integrity (Wood 1997; Taylor & Lehmann 1998). Nucleotide excision repair (NER) removes bulky DNA adducts, while base excision repair and mismatch repair correct spontaneous mutations arising from cellular replication errors and oxidative damage. Several groups have investigated possible carcinogenic inhibitory effects of HBx on NER (Prost *et al*. 1998; Groisman *et al*. 1999; Jia *et al*. 1999). This is likely to be the result of physical interaction between HBx and the NER apparatus (Lee *et al*. 1995; Capovilla *et al*. 1997; Becker *et al*. 1998). A study using yeast two hybrid analysis revealed binding by HBx to a protein termed X-associated protein 1 (XAP-1) (Lee *et al*. 1995). XAP-1 was homologous to a simian damaged DNA binding (DDB) protein and binding of XAP-1 by HBx was subsequently shown to correlate with inhibition of hepatocyte cellular DNA repair (Becker *et al*. 1998).

Further support for a role of HBx in DNA repair during

hepatocarcinogenesis comes from investigations on HBx interaction with p53. Transcriptional activation by p53 is thought to involve direct interaction of p53 with the general transcription factors, TFIID and TFIIH (Wang *et al*. 1995b). The XPB and XPD helicase components of TFIIH interact with p53 and are also required for transcription-coupled NER. It is thought that the association of p53 with TFIIH-coupled XPB and XPD normally inhibits transcription at damaged sites and initiates the formation of repair complexes. HBx alters the interaction of p53 with XPB and XPD (Wang *et al*. 1995b), and also inhibits p53-dependent DNA repair efficiency (Prost *et al*. 1998; Jia *et al*. 1999). A study to assess the effects of HBx expression on accumulation of mutations *in vivo* in transgenic mice was recently performed (Madden *et al*. 2000). The results demonstrated that HBx does not significantly increase the occurrence of spontaneous mutations in the transgenic mice. However, these spontaneous mutations result from replication errors or oxidative metabolism, and are repaired by mismatch repair and base excision repair. Present evidence indicates that HBx has its effects on NER, which is required for the removal of bulky DNA adducts. Thus, exposure to adduct-forming DNA-damaging agents may be required for HBx to have an effect on the accumulation of potentially carcinogenic mutations.

The importance of an effect of HBx on DNA repair that is required for viral replication is not established. A possibility is that HBx is required for the conversion of partially double stranded DNA to cccDNA during an initial step of HBV replication. If this is a biologically important action of HBx, it is not clear why avian hepadnaviruses would not require HBx for repair of their partially double stranded genomes.

HBV sequence variations that are associated with HCC

HBV mutations are associated with a variety of clinical manifestations of acute and chronic infection with the virus. These include a fulminant course, resistance to lamivudine and interferon therapy, vaccine escape and a low replication rate in chronic carriers (reviewed in Carman 1995; Günther *et al*. 1999). Several mutations of HBV have been found to be associated with HCC, which may be important to the mechanisms underlying hepatocarcinogenesis.

Mutations of the precore region of HBV have been the most extensively studied (Günther *et al*. 1999). These mutations usually inactivate translation of the precore region and consequently inhibit the synthesis of HBeAg. The precore mutations include substitutions that result in the formation of premature stop codons or missense mutations, as well as frameshift mutations and changes to the precore initiation codon. The G to A transition at position 1896 is the most frequently observed precore mutation and converts a codon in the precore region to a premature stop codon. This mutation may also affect the structure of the HBV encapsidation signal, ε, which comprises a stem loop structure that is formed by intrastrand base pairing in the HBV pregenomic RNA. In the normal stem-loop structure of the encapsidation signal, the base at 1896 pairs with the base at 1858. In genotypes A and F, there is a C at position 1858, and the 1896 G to A transition is thought to destabilize ε and be incompatible with HBV replication (Laskus *et al*. 1994; Lindh *et al*. 1995; Rodriguez-Frias *et al*. 1995). In genotypes B to E, where there is a U residue at 1858 of pregenomic HBV RNA, the 1896^{A} mutation does not destabilize ε function (Norder *et al*. 1994). Initial studies on the nucleotide sequence of the precore/core sequence in patients with HCC demonstrated that mutations in this region are not associated with a course of chronic infection that is complicated by HCC (Clementini *et al*. 1993; Minami *et al*. 1996). The 1896^{A} mutation is uncommonly observed amongst predominating genotype A isolates from South Africa (Kramvis *et al*. 1997). A recent study on patients from Hong Kong and China revealed the 1896^{A} precore mutation in 22.8% of HBV chronic carriers and in 5.6% of patients with acute HBV infection (Zhong *et al*. 2000). Moreover, the mutant HBV DNA was integrated into malignant hepatocyte DNA in 65% of HCC cases studied. Although the mutation was detected commonly in HCC, it was suggested that the 1896^{A} mutation may not be hepatocarcinogenic *per se*, but that it is selected during the course of chronic infection leading to HCC.

Analysis of sequence variation in the precore region of HBV in patients from southern Africa revealed that a missense G to T transversion at position 1862 occurs in asymptomatic HBV carriers (Kramvis *et al*. 1997) and more commonly in patients with HCC (Kramvis *et al*. 1998). Nucleotide 1862 is within the sequence encoding the signal peptide of the HBeAg precursor, and also the bulge sequence of ε that is required for priming of reverse transcription. Thus, the point mutation at 1862 may interfere with processing of the HBeAg precursor priming as well as packaging of HBV pregenomic mRNA. Compromised HBV replication that results from the 1862 mutation may also promote HBV integration and hepatocarcinogenesis.

Analysis of the data from several studies reveals that, with one exception, changes in the core gene of HBV are similar in asymptomatic HBV chronic carriers and in

those who develop HCC (Hosono *et al*. 1995; Pollicino *et al*. 1995; Minami *et al*. 1996; Günther *et al*. 1999). Substitution of proline for serine at amino acid 181 in the deduced core amino acid sequence occurs most commonly in HBV-infected individuals who develop HCC or fulminant infection.

The proteins encoded by the surface open reading frame are potentially oncogenic by mechanisms that involve activation of transcription and modulation of cell signalling pathways (discussed above) (Kekule *et al*. 1990; Hildt *et al*. 1996; Alka *et al*. 2000). Mutations to the surface ORF are likely to be most important for the evasion of the host immune surveillance but their direct role in hepatocarcinogenesis is presently unclear (Günther *et al*. 1999). HBV escape from the host immune response, as a result of surface mutations, may favour the clonal expansion of hepatocytes expressing the mutant protein (Zhong *et al*. 1999). Substitution of glycine with arginine at position 145 of the surface protein is a particularly common mutation that is associated with immunoevasion. This mutation is frequently observed in HBV DNA from Asian patients with HCC (Zhong *et al*. 1999).

HBx ORF and basic core promoter/enhancer II element mutations occur commonly in HBV chronic carriers with HCC from southern Africa (Baptista *et al*. 1999) and China (Hsia *et al*. 1996). Analysis of HBV sequences isolated from serum revealed that the prevalence of paired 1762 A to T (1762^T) and 1764 G to A (1764^A) mutations was 66% in the serum from patients who had HCC and in 11% of asymptomatic carriers from southern Africa (Baptista *et al*. 1999). In liver tissue from southern African patients with HCC, the 1762^T and 1764^A mutations were found in 15 of 28 tumours (54%) and in 4 out of 10 (40%) adjacent nontumour samples. In a similar study on patients from China, 1762^T and 1764^A mutations were found in 17 of 20 HCC samples (85%) and also in 17 of 20 adjacent nontumour tissue samples (Hsia *et al*. 1996). These mutations were also detected in 2 of 18 serum samples (11%) of Chinese HBV carriers without HCC. Deletion mutations that occur in the 3′ end of the HBx ORF, resulting in HBx truncation, have also been reported to occur more commonly in patients with HCC (Hsia *et al*. 1997). 1762^T and 1764^A mutations may result in amino acid substitutions in HBx, and also changes in the binding of transcription factors to the overlapping basic core promoter/enhancer II element. In *HBx*, 1762^T and 1764^A mutations result in significant and possibly carcinogenic changes to amino acids 130 (lysine to methionine) and 131 (valine to isoleucine). Several studies have demonstrated that 1762^T and 1764^A mutations decrease the concentration of pre-C mRNA and consequently HBeAg (Buckwold *et al*. 1996; Moriyama *et al*. 1996; Buckwold *et al*. 1997; Scaglioni *et al*. 1997). However, the influence of these mutations on HBV replication is not clear. Thus the role of 1762^T and 1764^A mutations in the pathogenesis of HCC has not been established.

Insight into the mechanisms of hepatocarcinogenesis effected by HBx mutations was revealed in a study on a group of mutant HBx sequences that promotes cell growth and inhibits apoptosis (Sirma *et al*. 1999). Expression of wild-type HBx was shown to cause apoptosis and arrest cells in the G1/S stage of cell cycle progression. The reasons for the discrepancy between these results and those reported by Benn & Schneider (1995), who showed that expression of wildtype HBx deregulates cell cycle checkpoint controls, are currently unclear. Nevertheless, mutant versions of *HBx*, derived from tumours with integrated HBV sequences, displayed defective growth-suppressive properties (Sirma *et al*. 1999). Mapping studies revealed that the antiproliferative properties of HBx correspond to the transcriptional activating region of the protein. It was suggested that during multistep hepatocarcinogenesis, there is selection for hepatocytes that express HBx mutants with defective transcriptional activating and antiproliferative properties. This hypothesis is supported by the observation that intact HBx is rarely found in HBV integrants of HCC (Robinson 1994).

Indirect hepatocarcinogenicity

In all geographical regions, the majority of HCCs coexist with cirrhosis (Kew & Popper 1984). The association is generally less close in black African populations. For example, cirrhosis is present in 54% of southern Africans with HBV-related HCC. The average global figure for coexistence of cirrhosis and HCC is 70–75% and the tumour coexists with chronic hepatitis in a further small percentage of patients or the chronic fibrotic hepatic disease that complicates longstanding hepatic venous congestion. Both HCC and cirrhosis result from persistent HBV infection, and cirrhosis is therefore not necessarily implicated in the aetiology of HCC. However, the evidence that chronic necroinflammatory hepatic disease plays a role in the pathogenesis of HCC is provided by the observation that the lifetime relative risks for developing HCC in HBV chronic carriers with cirrhosis is higher than in a carrier without cirrhosis (Beasley *et al*. 1981; Beasley & Hwang 1984). Studies on the pre S/S transgenic mouse model (Chisari *et al*. 1989) also support the idea that HBV-induced chronic

necroinflammatory hepatic disease *per se* may lead to neoplastic transformation. These mice overproduce large envelope (PreS1) protein, which accumulates in the endoplasmic reticulum of hepatocytes and causes severe injury to these cells. The resulting inflammation, regenerative hyperplasia, and transcriptional deregulation progress to neoplastic transformation.

A number of mechanisms has been proposed for the pathogenesis of HCC that results from chronic necroinflammatory disease. Hepatocytes normally divide extremely infrequently and the recurring cycles of hepatocyte necrosis and regeneration that characterize virus-induced necroinflammatory disease markedly increases hepatocyte turnover rate. Increased proliferation is required for hepatocarcinogenesis (Webber *et al*. 1994), although proliferation *per se* does not transform cells. By increasing hepatocyte turnover rate, chronic necroinflammatory hepatic disease greatly enhances the risk of a cell being initiated and of an initiated cell progressing to a fully malignant phenotype. It thus acts as a potent tumour promoter. Three putative mechanisms are involved. First, single-stranded chromosomal DNA, formed during cellular DNA replication, is more susceptible than double-stranded DNA to HBV DNA integration. Increased intracellular activity of topoisomerase I, which results from hepatocyte proliferation, may predispose to cellular integration of HBV DNA (see above) (Wang 1985). Mutations resulting from spontaneous errors of replication or the effects of chemical carcinogens are more likely to occur in proliferating than in quiescent hepatocytes. A regenerative response is obligatory for the initiating action of a liver carcinogen (Cayana *et al*. 1978), and the greatest transformation sensitivity occurs during the G1 to early S phase of the cell cycle (Rabes *et al*. 1986). Second, an accelerated rate of cell division allows less time for mutated cellular DNA to be repaired before the cell divides again, and in the presence of inhibited apoptosis, this fixes the abnormal DNA in the daughter cells. This results in the accumulation over time of a series of mutations that is essential for tumour formation. Inhibitory effects of HBx on programmed cell death and DNA repair (see above) may contribute to this mechanism by compromising apoptosis in response to DNA damage. Finally, an increased rate of hepatocyte division allows selection and clonal expansion of premalignant or initiated hepatocytes (Sugimara 1992). In experimental chemical carcinogenesis models, initiated hepatocytes may have either a proliferation advantage over normal cells or might be more responsive to promoters of carcinogenesis (Cerutti 1988).

Hepatic fibrosis, which is part of the chronic necroinflammatory hepatic disease, distorts the lobular architecture of the liver. This modifies cell-to-cell and cell-to–extracellular matrix interactions and may interfere with the putative 'streaming' of maturing hepatocytes from the portal to the centrilobular areas. It has been suggested that this contributes to the loss of cell growth control (Craig *et al*. 1991; Davis & Kresina 1996). Moreover, changes in the microcirculation of the liver secondary to fibrosis may channel oncogenic agents to selected groups of hepatocytes.

Hepatic inflammation *per se* results in the local production of oxygen reactive species that are produced by infiltrating mononuclear cells (Freeman & Crapo 1982). Oxygen reactive species exert several effects that may contribute to hepatocarcinogenesis. The formation of 8-hydroxy-2′-deoxyguanosine DNA adducts is mutagenic and may induce strand breaks in hepatocyte DNA, which would facilitate HBV DNA integration. Oxygen reactive species may damage intracellular organelles by peroxidation of membrane lipids and modify the cysteine sulphydryl groups that are important to the conformation-dependent activity of several proteins. These include transcriptional regulators, e.g. Fos, Jun and NF-κB, as well as components that are required for the activation of the antioxidant response element.

WHV-and GSHV-related HCC is associated with active hepatic necroinflammation but not with cirrhosis (Gerin *et al*. 1986; Marion *et al*. 1987; Popper *et al*. 1987). These animals also show hyperplastic and preneoplastic nodules that progress to HCC, similar to that seen in chemical carcinogenesis but not described in HBV-related HCC in humans. It is not clear whether the inflammation has a promoting effect or is a response to tissue injury caused by the rapidly growing tumour.

Finally, cirrhosis may predispose to HCC formation through its effect on antitumour immunity in the liver. NK cells and $CD56^{+}$T cells have been shown to play an important part in antitumour immunity in the liver (Satoh *et al*. 1996; Cui *et al*. 1997). These cells are reduced in the cirrhotic liver (Kawachi *et al*. 1995; Kawarabayashi *et al*. 2000) and this offers a further possible explanation for the frequency with which HCC develops in cirrhosis.

Conclusions

HCC that results from chronic HBV infection is a malignancy with a poor prognosis and is among the most prevalent tumours in many of the populous regions of the world. Application of modern methods of molecular biology has greatly facilitated our understanding of

the mechanisms of HBV infection and the processes underlying HBV-induced hepatocarcinogenesis. Effective vaccines against HBV are now available and HBV immunization is compulsory in many countries. However, economical and logistical reasons make implementation of HBV immunization programmes problematic in many of the endemic areas. Efficient delivery of vaccination thus remains an important priority that will define the objective of eliminating HBV-related HCC well into the future.

There are several oncogenic mechanisms that are thought to underlie HBV-associated HCC. These include direct effects of the virus as well as indirect consequences of the chronic necroinflammatory disease that often accompanies HBV infection. Potentially oncogenic direct effects of HBV include the *cis*-activation of hepatocyte growth regulatory genes by integrated HBV DNA. Although WHV is frequently integrated at the *c-myc* or *N-myc* loci in WHV-related HCC, oncogenic insertional mutagenesis of hepatocyte growth regulatory genes by HBV appears to be rare. This suggests that hepatocarcinogenic mechanisms differ according to infected species and/or species of infecting hepadnavirus. Effects of HBx on transcriptional activation, apoptosis and DNA repair implicate this protein in the aetiology of HBV-associated HCC. The reported effects of HBx on hepatocyte transformation have been unusually diverse and, in some cases, contradictory. The step(s) in the HBV life cycle that requires the participation of HBx has also remained elusive and contributed to the frustration in understanding HBV-induced hepatocarcinogenesis. Future definition of the role of HBx *in vivo* in the viral life cycle is a research priority that should go some way to understanding the mechanism of HCC that is associated with chronic HBV infection.

Acknowledgements

Financial support from the Cancer Association of South Africa, the South African Poliomyelitis Research Foundation, Medical Research Council and National Research Foundation is gratefully acknowledged.

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